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PHARMACOGNOSTICAL EVALUATION AND ANTHELMENTIC ACTIVITY OF *SWERTIA ALATA* ROYLE

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ABSTRACT: Objective: In the present investigation, pharmacognostical, physicochemical characteristics, thin layer chromatography and anthelmintic activity of different extracts of *Swertia alata* Royle were studied. **Methods:** Different types of preliminary and phytochemical analysis and quantitative estimation have been done along with HPTLC fingerprinting. Two concentrations (30 mg/mL and 60 mg/mL) of different extracts of *S. alata* were used for anthelmintic activity against Indian earthworm *Pheretima posthuma*. **Results:** The microscopic study revealed the presence of lignified vessels and cruciferous stomata. The chemical tests showed the presence of glycosides, saponins, tannins, proteins and steroids. HPTLC fingerprinting of different extracts showed number of spots due to presence of various phytochemical compounds. The results of anthelmentic activity were expressed in terms of time of paralysis and time for death of worms. Piperazine citrate (10mg/mL and 30 mg/mL) was used as a reference standard and normal saline as a control group. **Conclusions:** These observations will be useful in evaluation of pharmacognostical and phytochemical standards to ensure the purity and quality of this plant. The anthelmintic activity of *S. alata* aerial extracts has therefore been demonstrated for the first time.

INTRODUCTION: Plants have been used medicinally for thousands of years by cultures all over the world. According to the World Health Organization, 80% of the world's population uses plant-based remedies as their primary form of healthcare [1]. A majority of the world's population in developing countries still relies on herbal medicines to meet its health needs. Even in areas where modern medicine is available, the interest on herbal medicines and their utilization have been increasing rapidly in recent years [2].

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Herbal medicine can offer an alternative to modern medicine in non life threatening conditions, providing they are of adequate quality and safety, and are used in an appropriate manner by suitable individual [3]. For preparation of herbal formulation or using herbs as medicine, identification and standardization are rudimentary. Identification involves study of morphological and microscopical parameters of plant and standardization of herbal drugs includes study of physical and chemical parameters. These studies help in identification and quality assurance of the starting material and to ensure the safety and efficacy of herbal products [4]. Infection with helminthes, or parasitic worms, affect more than two billion people worldwide. In regions of rural poverty in the tropics, where prevalence is greatest, simultaneous infection with more than one type of helminth is common [5].

The treatment of these infections in the 21st century is mostly through the use of modern synthetic agents [6]. Anthelmintics are the drugs that either kill (Vermicide) or expel (Vermifuge) infesting helminthiasis [7]. The regular intake of synthetic drugs may produce dependency, where there is less or no possibility of abuse in case of herbal plants. They provide pure and potentially active compounds [8].

Swertia alata Royle is a perennial herb 1-2 feet tall, with a straight glabrous green erect stem. Leaves are ovate, entire and oppositely arranged. It grows commonly in temperate west Himalayas 4000 - 6000 ft particularly from Kashmir to Kumaon, Mussorrie, Dehradun and Nainital region [9]. It is one of the common adulterant of *S. chirata* which is widely used in indigenous system of medicines as a bitter tonic, febrifuge, laxative and antimalarial [10]. Phytochemically, it contains swertisin, swertiamarin, bellidifolin and oleanolic acid [11].

MATERIAL AND METHODS:

Plant Material: The dried plant material was supplied by Almas Pharmaceutical Ltd, Uttar Pradesh and identified by Dr. H. B. Singh, NISCAIR (National Institute of Science Communication and Information Resources) Pusa Gate, New Delhi. The voucher specimen (NISCAIR/RHMD/2013/2185/191) of the test drug has been deposited in the herbarium of NISCAIR for future reference.

Preparation of Extract: The aerial parts of *S. alata* were powdered in a mixer grinder. The powdered aerial parts packed in a paper bags and stored in air tight container until use. The coarse powdered material of *S. alata* was extracted successively by petroleum ether, chloroform, ethanol and distilled water. The extracts were concentrated under reduced pressure. Each time before extraction with next solvents, the coarse powdered material was dried in hot air oven below 50 °C. The extracts were stored at cool place in dark until use.

Macroscopic and Microscopic Analysis: Macroscopic studies were done by using simple microscope. Aerial parts were subjected to morphological evaluation for colour, odour, taste, shape and texture [12]. The leaves and roots were separated from the other parts of the plant (stem and shoot of leaf) cleaned manually and kept over dry plastic sheet to investigate different organoleptic features. The magnifying glass and scale were used to measure the parameters like morphology, length, width *etc*. The anatomical studies were performed on aerial parts of plant. Powder (# 60) of the dried leaves, stems and roots was used for the observation of powder microscopical characters. All the parts were cut across sectioned with free hands. Various types of chemical tests were performed for identification of various structures. Chloral hydrate, hydrochloric acid and phloroglucinol were used for detection of various structures. Transverse sections and slides of powdered *S. alata* were prepared and permanent staining was done to study the anatomical features.

Leaf Constants: To evaluate the drug, various leaf constants like stomatal index, stomatal number, palisade ratio, vein islet number and vein termination number has been done [13].

Physico-Chemical Analysis: Physico - chemical parameters of the powdered drug such as total ash, water-soluble ash, acid-insoluble ash and sulphated ash were determined. Alcohol and water-soluble extractive values were determined to find out the amount of water and alcohol soluble components. The moisture content was detected by loss on drying method [14, 15].

Fluorocesence Analysis: The powdered drug was taken as such and treated with various solvents and subjected to fluorescence analysis. Observations were made under day light, UV light of short and long wavelengths, separately [16].

Qualitative Analysis: The preliminary qualitative phytochemical identification was carried out by using different phytochemical tests to check the presence of different phytoconstituents like steroids, flavanoids, phenolic, saponins and terpenes was qualititatively estimated [13].

HPTLC Analysis: The Sample (10µL each) was applied in the form of bands on pre-coated silica gel 60GF254 aluminium sheets (20x10 cm) with the help of Linomat V applicator attached to CAMAG HPTLC system, which was programmed through WINCATS software 24.

Development of Chromatogram: After the application of spots, prepared plates were developed in previously saturated twin trough chamber (20x10 cm) in linear ascending direction with solvents at specified time.

Detection of Spots: The developed plates were dried by hot air to evaporate the solvents from the plate. The developed plate was sprayed with anisaldehyde sulphuric acid as spraying reagent and dried at 100 °C in hot air oven for three minutes. The plate was kept in photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images under UV light at 254 nm and 334 nm, respectively. The R_f values and fingerprint data were recorded by WINCATS software [17].

Collection and Authentication of Worm: Indian adult earthworms (*Pheretima posthuma*) were collected from the water logged areas of soil and washed with normal saline solution to remove all the faecal matter and adhering dirt. The worms were identified and authenticated by Dr. Dileep K. Singh, Associate Professor (Zoologist) from Department of Zoology, University of Delhi, New Delhi, India.

Anthelmentic Activity of Different Extracts: Anthelmentic activity of petroleum ether, chloroform, ethanolic and aqueous extract of *S. alata* aerial parts was evaluated on Indian earthworms. The samples were prepared by dissolving 300mg and 600mg of each extract in 1.0mL DMSO and made the volume up to 10mL with normal saline solution to prepare 30mg/mL and 60mg/mL concentration. Eleven groups of Indian earthworms, each containing two earthworms of approximately equal size were used for the study. Three groups were tested with each extract of different concentrations (30mg/mL and 60mg/mL) and the other three groups were treated with piperazine citrate (30mg/mL), as a reference standard [18].

One group was treated with normal saline solution and used as control group. The groups were observed for paralysis time and death time for each earthworm [19, 20]. The paralysis time was said to occur when there is no sort of movement except when shaken vigorously and death time was recorded after ascertaining that worms neither moved when given external stimuli nor dipped in warm (50 °C) water [8]. All experiments were repeated thrice. The mean and SEM were analysed statistically by ANOVA followed by Turkey's test, $P < 0.05$ being considered as significant.

RESULTS:

Macroscopy Studies: It is assumed that morphological evaluation of any plant drug is considered to be the primary step for establishing its quality control profile. Proper authentication of a drug depends almost entirely on morphological characters. The morphology of leaf, stem and root of *S. alata* is given in **Table 1**.

TABLE 1: MORPHOLOGICAL EVALUATION OF AERIAL PARTS OF *S. ALATA*

S. no	Character	Leaf	Stems	Roots
1.	Colour	Greenish black	Brown	Brownish black
2.	Odour	Characteristic	Characteristic	Characteristic
3.	Taste	Very bitter	Very bitter	Very bitter
4.	Shape	Ovate	Cylindrical	Fibrous
5.	Texture	Brittle	Hard	Hard

Microscopical Studies: The microchemical test for powdered form of *S. alata* was carried out to identify the composition of microscopical structures and the observation was given in **Table 2**.

TABLE 2: MICROCHEMICAL TEST

Chemical test	Observation	Inference
Drug + phloroglucinol + Hcl	Pink coloured walls of cork	Lignin present
Drug + few drops of water	No swelling observed	Mucilage absent
Drug + Iodine	No blue colouration	Starch absent

The transverse section of stem reveals that a single layered epidermis, with cubical cells was present, which was covered externally by a striated cuticle. The sub epidermal collenchymas were 2-3 layered followed by a wide zone of parenchyma of 8-10 layers. The pericycle was indistinct. The phloem

was present on both sides of xylem. There was a thin layer of cambium between the phloem and xylem. The vessels were mostly reticulate and pitted. The parenchymatous pith was also present (**Fig. 1** and **2**).

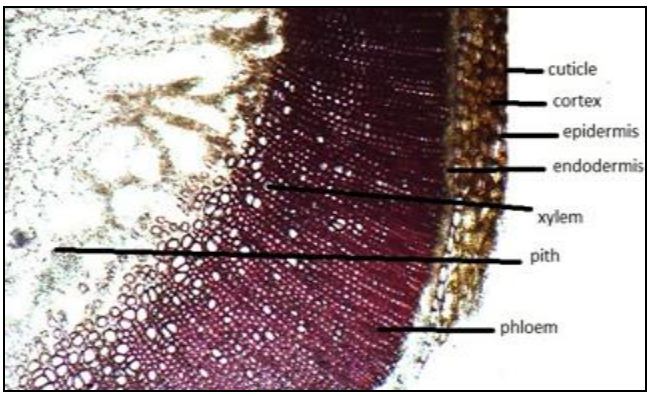

FIG. 1: T. S. OF STEM (40X)

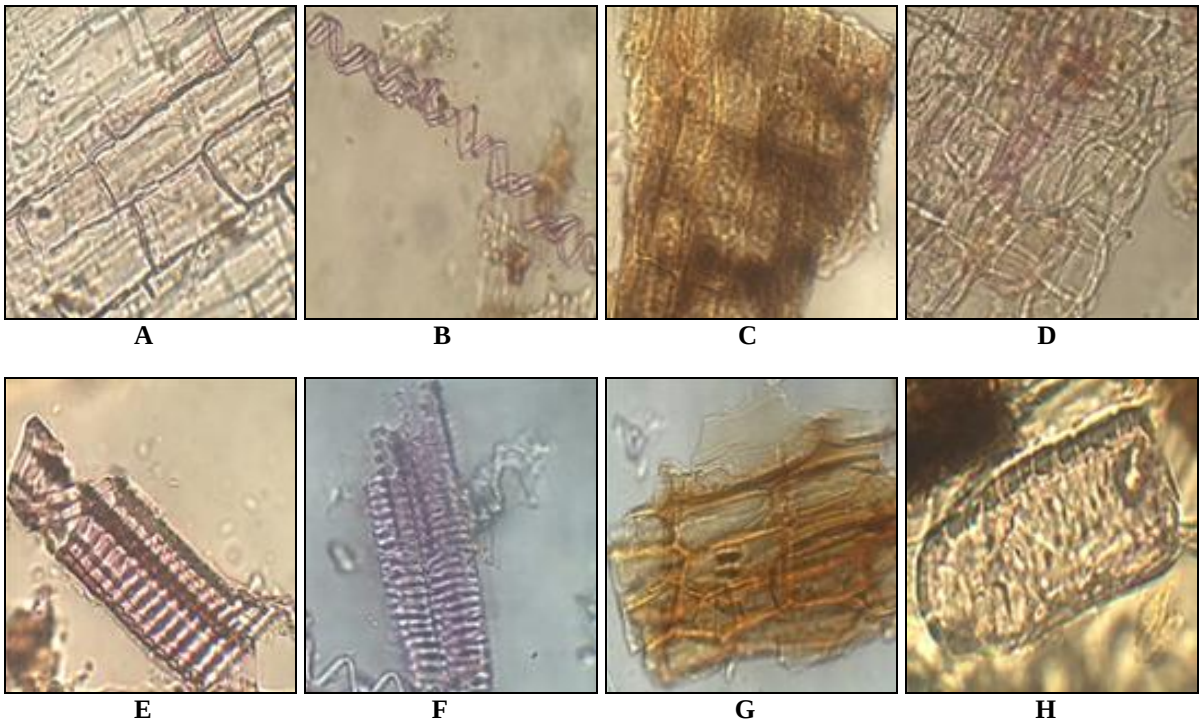

FIG. 2: POWDERED MICROSCOPY OF STEM (40X) : (A) CORK CELLS (B) ANNULAR VESSELS (C) MEDULLARY RAYS (D) CORK CELLS IN SURFACE VIEW (E) PITTED VESSEL (F) SPIRAL VESSEL (G) CORK CELLS CONTAINING BROWN COLOUR (H) STONE CELL

Tranverse section of leaves reveals that it was isobilateral, irregularly elevated with two lateral laminar extensions. Upper epidermis embedded with stomata and it was devoid of trichomes with cruciferous stomata, loosely arranged parenchyma and vascular bundles were present in centre (**Fig. 3** and **4**).

Diagrammatic transverse section of root was circular in outline, showing outermost single layered epidermis, showing parenchymatous cortex and successive alternate more or less concentric rings of secondary vascular tissue occupying the major central area of the section. It concludes that there was a single layered epidermis with 4 to 6 rows of parenchymatous layers with the innermost endodermis. The pericycle was thin walled and showed concentric rings of xylem alternating with narrow parenchymatous band and medullary rays connecting them, alongwith presence of protoxylem and metaxylem with phloem (**Fig. 5** and **6**).

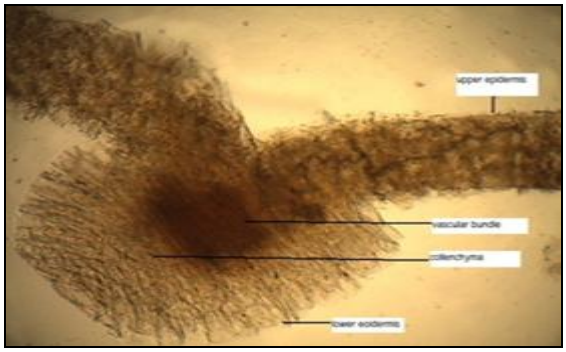

FIG. 3: TRANSVERSE SECTION OF LEAF (10X)

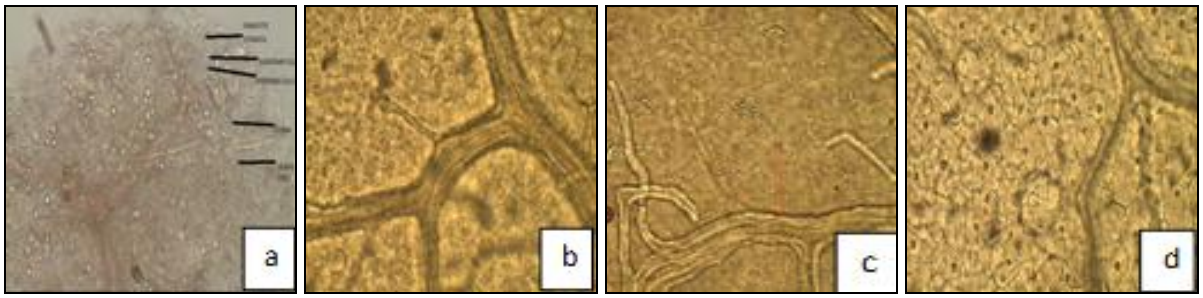

FIG. 4: POWDERED MICROSCOPY OF LEAF (40X): (a) STOMATA OF LEAF (b) VEIN LETS (c) VEIN TERMINATIONS (d) EPIDERMAL CELLS BENEATH PALISADE CELLS

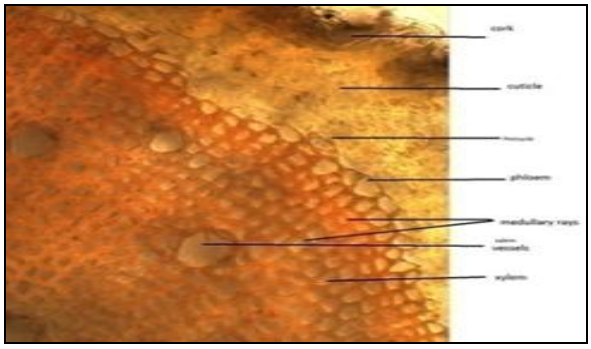

FIG. 5: T. S. OF ROOT (40X)

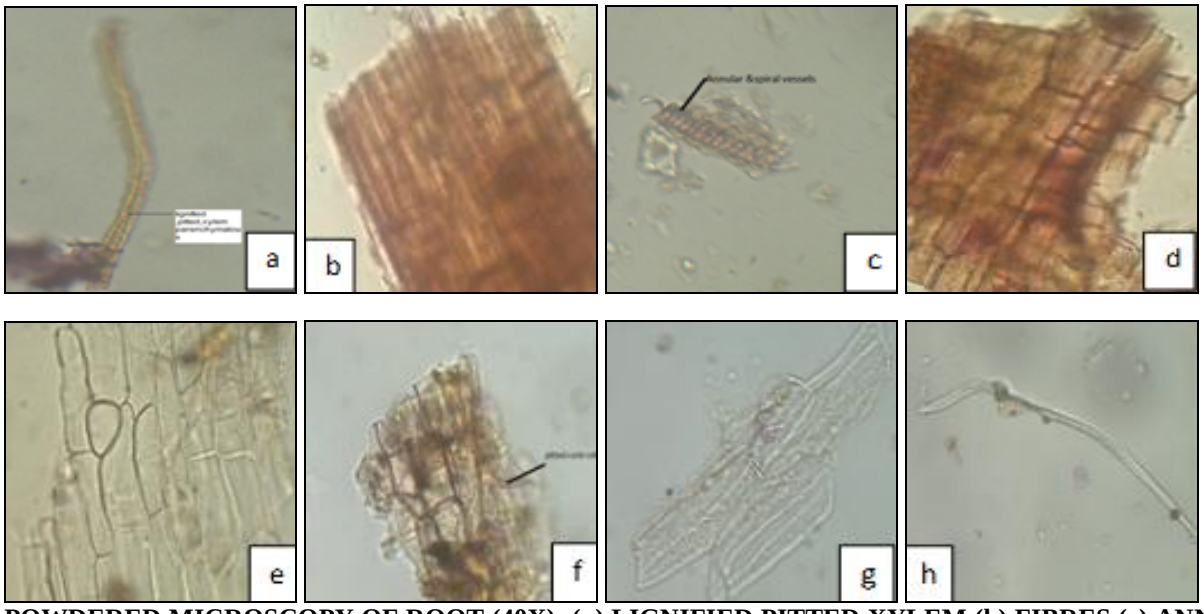

FIG. 6: POWDERED MICROSCOPY OF ROOT (40X): (a) LIGNIFIED PITTED XYLEM (b) FIBRES (c) ANNULAR AND SPIRAL VESSEL (d) LIGNIFIED CORK CELLS (e) RADIALLY CUT FRAGMENT OF MEDULLARY RAYS (f) FRAGMENT OF PITTED CORK CELLS (g) VESSEL (h) FIBRES

Leaf Constants: Various leaf constants (microscopical constants/quantitative microscopy) are used for standardization of leafy drugs and in detection of adulterants. In leaf constants, stomatal index and palisade ratio were found to be 13.04-14.81 and 1 - 4, respectively. Whereas, vein islet and vein termination number were ascertained as 23 - 25 and 46 - 48, respectively. Stomatal number was found to be 24-26.

Physicochemical Analysis: The physicochemical parameters like ash values, inorganic element, extractive values and moisture content were important to determine the purity of drug. The ash content of drug also showed presence of calcium, magnesium and sulphate while absence of sodium, potassium and phosphate types of inorganic compounds. Inorganic elements (Fe, Zn, K and Ca) have been measured by Atomic Absorption spectroscopy in plant samples and the results are recorded in the **Table 3**.

TABLE 3: PHYSICO-CHEMICAL PARAMETERS

S. no	Parameters	Percentage
1.	Ash values	
	Total ash	7.47
	Acid insoluble ash	5.56
	Water soluble ash	1.57
	Sulphated ash	0.34
2.	Extractive values (gm %)	
	In cold alcohol	7.2
	In hot alcohol	8
	In hot water	10
3.	Moisture content	0.15%
4.	pH	
	In 1% aqueous solution	7.5
	In 10% aqueous solution	7.2
5.	Successive extractives	
	Petroleum ether extract	1.968%
	Chloroform extract	1.613%
	Ethanol extract	7.963%
	Aqueous extract	9.012%
6.	Test for extraneous material	
	Foreign matter	0.1%
	Sand &silica	Not visible
	Insect infestation	Nil
	Rodent contaminations	Nil
7.	Inorganic elements	
	Fe	533.981
	Zn	52.666
	K	0.88
	Ca	.11

The crude drug was screened for the presence of microbial contamination. Total aerobic microbial count, Enterobacteriaceae and fungal count, pesticide residue, as per the method laid down in Indian Pharmacopoeia (2010). The results are recorded in **Table 4**.

TABLE 4: MICROBIAL CONTAMINATION, HEAVY METALS, TEST FOR AFLATOXIN AND PESTICIDE RESIDUE

Microbial contamination	Observation	Limit
Total aerobic microbial count	Less than 10	10^5
Enterobacteriaceae	Less than 10	10^3
Total fungal count	Less than 10	10^1
Pesticide Residue	Observation	Limit (ppm)
DDT	Not detected	0.0050
HCH (Alpha & Beta)	Not detected	0.0050
Endosulfan Alpha endosulfan Beta endosulfan Endosulfan sulphate	Not detected	0.0050
Malathion	Not detected	0.0050
Parathion	Not detected	0.0050
Heavy metal	Observation	Limit (ppm)
Arsenic	Not detected	0.2
Lead	Not detected	0.2
Mercury	Not detected	0.2
Cadmium	Not detected	0.2
Aflatoxin	Observation	Limit (ppb)
B_1	Not detected	1.0
B_2	Not detected	1.0
G_1	Not detected	1.0
G_2	Not detected	1.0

Fluorescence Characteristics

Fluorescence Characteristics of Powdered Drug: The powder of *S. alata* was treated with routinely used reagents and characteristic changes were observed and summarized in **Table 5**.

Fluorescence Characteristics of Extracts: Fluorescence characteristics of the extracts were observed in day light as well as in ultraviolet radiation. The results were recorded in **Table 6**.

Qualitative Phytochemical Investigations: All the extracts were subjected to preliminary phytochemical screening and the results were recorded in **Table 7**.

HPTLC Fingerprinting: HPTLC profile of different extracts of *S. alata* are found as per **Table 8** when developed TLC plates were observed under UV light, iodine chamber and after derivitization with 15% ethanolic sulphuric acid followed by heating at 105 °C for 15 min (**Fig. 7**).

TABLE 5: FLUOROSENCE CHARACTERISTIC OF POWDERED DRUG

S. no	Chemical treatment	Ordinary light	UV long WL	UV short WL
1.	Powdered Drug	Light green	Purplish black	Dark green
2.	Powdered Drug treated with distilled water	Light green	Purplish black	Dark green
3.	Powdered Drug treated with 50% HCl	Brown	Black	Greenish black
4.	Powdered Drug treated with 50% H_2SO_4	Greenish black	Black	Black green
5.	Powdered Drug treated with 50% HNO_3	Brownish green	Black	Yellowish
6.	Powdered Drug treated with conc. HCl	Brownish green	Black	Greenish black
7.	Powdered Drug treated with conc. H_2SO_4	Black	Black	Black
8.	Powdered Drug treated with conc. HNO_3	Brown	Black	Greenish
9.	Powdered Drug treated with pet ether	Green	Black	Blackish brown
10.	Powdered Drug treated with $CHCl_3$	Black	Black	Black
11	Powdered Drug treated with CH_3OH	Brown	Black	Black green
12.	Powdered Drug treated with ethyl acetate	Black	Purplish Black	Black
13.	Powdered Drug treated with 10% $fecl_3$	Yellowish green	Flourcent yellow	Yellowish brown
14.	Powdered Drug treated with 10% NaOH	brown green	Black	Blackish green
15.	Powdered Drug treated with ammonia	Green	Florocent green	Blackish green
16.	Powdered Drug treated with picric acid	Yellow green	Black	Yellow green
17.	Powdered Drug treated with Iodine	Brownish green	Black	Brown black
18.	Powdered drug treated with Glaciel acetic acid	Black	Purplish green	Blackish green
19.	Powdered drug treated with CH_3OH and NaOH	Black brown	Black	Yellow green

TABLE 6: FLUORESENCE CHARACTERISTICS OF EXTRACTS

S. no	Extract	Ordinary light	UV Short WL	UV long WL
1.	Petroleum ether extract	Yellowish brown	Dark greenish black	Dark brown
2.	Chloroform extract	Black	Greenish black	Black
3.	Ethanol extract	Yellowish brown	Greenish brown	Yellowish
4.	Aqueous extract	Purplish black	Black	Black

TABLE 7: QUALITATIVE PHYTOCHEMICAL INVESTIGATION

Test	Pet ether extract	Chloroform extract	Ethanolic extract	Aqueous extract
Alkaloids	-	-	-	-
Flavanoids	-	-	+	+
Tannins	-	-	+	+
Saponins	-	-	-	+
Glycosides	-	-	-	+
Steroids	-	+	+	+
Steroidal terpenes	+	-	+	-
Phenolic	-	+	+	+
Gums and mucilage	-	-	-	+
Carbohydrates	-	-	+	+
Test for iridoids	-	-	+	-

TABLE 8: SHOWING R_f VALUES AND NUMBER OF SPOTS

S. no	Extracts	Mobile phase (v/v/v)	No. of spots	R_f
1.	Petroleum ether extract	Hexane: Ethyl acetate (9:1)	8	0.41,0.51,0.55,0.59,0.67,0.73, 0.77,0.93
2.	Chloroform extract	Toluene: Ethylacetate: Formic acid,(8:2:0.1)	9	0.21,0.32,0.41,0.58,0.71,0.78,0.88, 0.95,1
3.	Ethanolic extract	Toluene: Ethyl acetate (9:2)	4	0.34,0.76,0.88,0.96
4.	Aqueous extract	Butanol: aceticacid: water (5:4:1)	7	0.14,0.19,0.35,0.42,0.54,0.82,1

Anthelmentic Activity: From the observation a dose dependent paralytic effect was observed and then the worms were died finally. The time of death of all the groups was recorded. Earlier although all extracts showed anthelmintic activity in a dose dependant manner but ethanolic extract appeared to be more effective than other extracts. The statistical analysis conducted with Graph Pad Prism software (Version 5.00, USA). All the results are tabulated in **Table 9**.

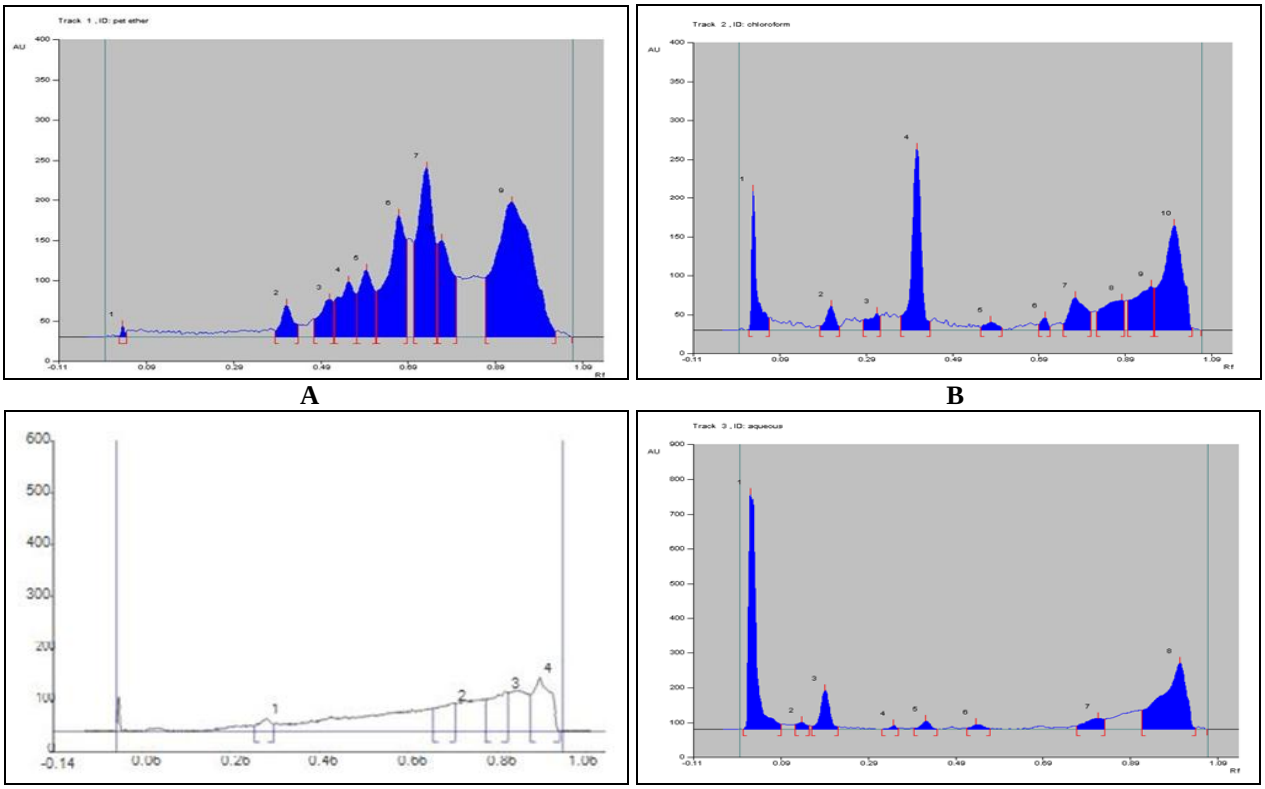

FIG. 7: HPTLC FINGERPRINTING OF (A) PETROLEUM ETHER EXTRACT (B) CHLOROFORM EXTRACT (C) ETHANOLIC EXTRACT (D) AQUEOUS EXTRACT

TABLE 9: ANTHELMINTIC ACTIVITY OF PETROLEUM ETHER, CHLOROFORM, METHANOL AND AQUEOUS EXTRACT OF AERIAL PARTS OF *S. ALATA*

Groups	Conc. (mg/mL)	Paralyzing time (min)	Death time (min)
Petroleum ether	30	50.05±0.05	73.7±2.2
	60	43.08±0.9	56.12±2.08
Chloroform	30	41.8±2.2	58.8±1.3
	60	34.08±0.05	44.72±1.5
Ethanol	30	28.1±0.9	39.16±0.9
	60	21.9±0.8	34.04±1.9
Aqueous	30	35.67±1.4	47.5±1.06
	60	27.11±2.09	41.06±0.9
Standard (Piperazine citrate)	10	19.44±1.6	28.04±1.9
	30	10.05±0.9	16.78±1.2
Control (Normal saline)	30	NA	NA

(Results expressed as mean ± SEM from three observations)

DISCUSSIONS: Despite the availability of hyphenated analytical techniques, identification and evaluation of plant drugs by pharmacognostical and physico-chemical parameter study is still more reliable, accurate and inexpensive. According to World Health Organization (WHO), the macroscopic and microscopic determination of the plant is the first step towards establishing its identity and purity and should be carried out before any tests are undertaken [13]. The estimation of moisture content of the drug is essential for evaluation of presence of bacteria and fungi count. Ash value, acid insoluble ash, water soluble ash and sulphated ash are useful for evaluating organic and inorganic impurities. It is difficult to obtain total purity of some organized drugs therefore presence of sand, pesticide, chemicals, microbes and toxic metals that may come in contact with the drug during the cultivation, collection, packing and storage should be in within limits [21].

Many phytochemical showed fluorescence when suitably illuminated with UV light [22]. If the substance themselves are not fluorescent, they may often be converted into fluorescent derivatives by applying different reagents hence some crude drugs

are often assessed qualitatively in parameter of pharmacognostical evaluation [23]. The change in appearance and colour were observed and depicted in **Table 5**. In the present study, Indian earthworms have been used for initial evaluation of anthelmintic activity, because of easy availability and their anatomical and physiological appearance with the intestinal roundworm parasites of human being [24]. The results of preliminary phytochemical test showed the presence of various phytochemical compounds in the plants which are known to have various therapeutic importance in medical sciences. Tannins might have anthelmintic activity by binding with free protein in gastrointestinal tract of the earthworm and cause death [25]. An anthelmintic drug can either act by causing paralysis of worm or by damaging cuticle, leading to partial digestion or to rejection by immune mechanism. They also interfere with the metabolism pathways of the worms [26, 27]. Although all the extracts exhibited anthelmintic activity but alcoholic extract of *S. alata* showed significant activity as compared with the reference compounds.

Table 9 depicts the time taken for paralysis and death of earthworms after treating with the test substances. It is observed that the ethanolic extract of plant was more potent than the reference control piperazine citrate. It caused paralysis followed by death of the worms at all tested dose levels. Potency of the extract was inversely proportional to the time taken for paralysis / death of the worms. The activity confirms the dose dependency nature of the extract. In the present study, we may conclude that the plant is also endowed with potential anthelmintic property. It would be interesting to isolate the possible constituents those are responsible for the anthelmintic activity.

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CONFLICT OF INTEREST: We declare that we have no conflict of interest.

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